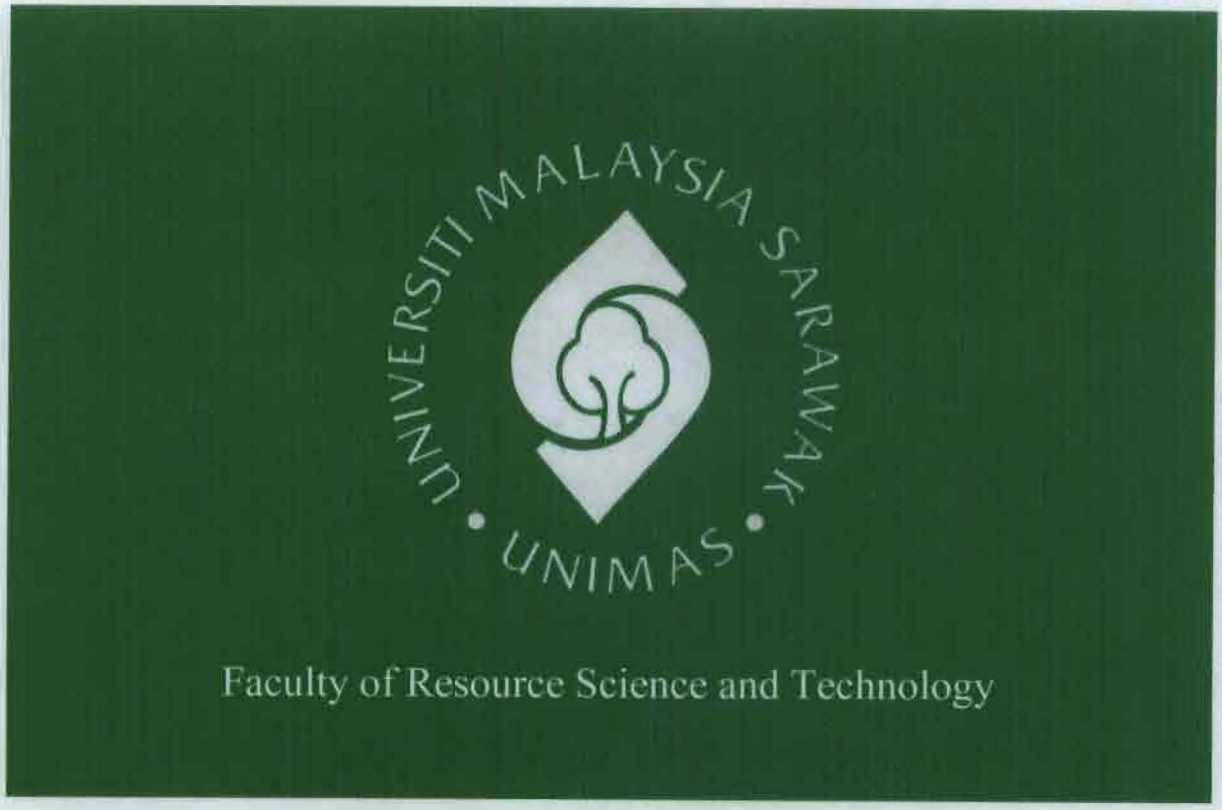

Distribution and Quantification of *Escherichia coli* and *Escherichia coli* O157: H7 in Organic Vegetables at Farm Level

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Bachelor of Science with Honours
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Distribution and Quantification of *Escherichia coli* and *Escherichia coli* O157: H7 in Organic Vegetables at Farm Level

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DECLARATION

I hereby declare that the thesis entitled "Distribution and Quantification of *Escherichia coli* and *Escherichia coli* O157: H7 in Organic Vegetables at Farm Level" submitted herewith for Degree of Bachelor of Science (Honours) at Universiti Malaysia Sarawak is my own work (except for the cited references) and has not been previously submitted by me at any other University for any purposes.

Name : LIM POH YIIN

Signature : Lim Poh Yiin

Date : 4 JULY 2013

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
AMP	Ampicillin
AST	Antimicrobial Susceptibility Test
bp	Base pairs
C	Chloramphenicol
CDC	Centers for Disease Control and Prevention
CFU	Colony-forming Unit
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
DAEC	Diffusely Adherent *E. coli*
DNA	Deoxyribonucleotides
DO	Doxycycline
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EMB	Eosin-methylene Blue
EPEC	Enteropathogenic *E. coli*
EtBr	Ethidium Bromide
ETEC	Enterotoxigenic *E. coli*
F	Nitrofurantoine
HUS	Hemolytic uremic syndrome
KF	Cephalothin

LB	Luria-Bertani
LBA	Luria-Bertani Agar
LPS	Lipopolysaccharides
MARI	Multiple Antibiotic Resistance Index
MHA	Mueller-Hinton Agar
ml	Milliliter
MPN	Most Probably Number
NaCl	Sodium chloride
OD	Optical Density
PCR	Polymerase Chain Reaction
S	Streptomycin
SOM	Skim Organik Malaysia (Malaysian Organic Scheme)
STEC	Shiga-toxin-producing *E. coli*
TBE	Tris-Borate EDTA
μg	Microgram
μL	Microlitre

LIST OF TABLES

LIST OF FIGURES

Distribution and Quantification of *Escherichia coli* and *Escherichia coli* O157: H7 in Organic Vegetables at Farm Level

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ABSTRACT

Organic vegetables are grown without the use of synthetic fertilizers and pesticides, thus they attract many consumers whom are concerned with their health. However, several researches had reported the presence of foodborne pathogens such as *Escherichia coli* (*E. coli*) and *Escherichia coli* O157: H7 in organic vegetables. The aim of this study is to detect and characterize *E. coli* as well as *E. coli* O157: H7 in raw organic vegetables from four organic farms located at Kuching and Siburan areas. Fifty one samples were collected and analyzed using Most Probable Number (MPN) supplemented with Polymerase Chain Reaction (PCR) method. *E. coli* was successfully isolated and enumerated from the organic vegetables samples. Characterization of nine of the *E. coli* isolates through antibiotics susceptibility test (AST) showed that five of them were multi resistant and possessed high risk of contamination by having Multiple Antibiotic Resistance (MAR) Index of 0.571 and 0.286. Fortunately, none of the samples tested harbored for pathogenic *E. coli* O157: H7.

Key words: *Escherichia coli*, *Escherichia coli* O157: H7, organic vegetables, antibiotics susceptibility tests, MPN-PCR

ABSTRAK

Sayur-sayuran organik ditumbuh tanpa penggunaan baja-baja sintetik dan racun perosak. Ini telah menarik perhatian daripada ramai pelanggan yang lebih prihatin terhadap kesihatan mereka. Bagaimanapun, patogen daripada makanan seperti Escherichia coli (E. coli) dan E. coli O157: H7 telah dilaporkan dalam pelbagai kajian tentang sayur-sayuran organik. Objektif kajian ini adalah untuk mengesan dan memperbahagikan ciri-ciri E. coli dan E. coli O157: H7 dalam sayur-sayuran organik mentah pada peringkat kebun. 51 sampel telah diperolehi dari 4 kebun organik di kawasan Kuching dan Siburan. Mereka telah dianalisis dengan kaedah MPN serta PCR. E. coli telah berjaya dikesan dan diasingkan daripada sampel-sampel tersebut. Pembahagian ciri-ciri E. coli juga telah dilakukan melalui ujian kecenderungan antibiotik (AST) dan ujian tersebut telah menunjukkan sebahagian daripada mereka adalah cenderung terhadap pelbagai antibiotik. Mereka juga didapati membawa risiko yang tinggi dalam pencemaran makanan, dengan mempunyai Indeks Ringtangan Pelbagai Antibiotik (MARI) bernilai 0.571 dan 0.286. Namun, antara semua sampel sayur-sayuran organik yang dikaji, E. coli O157: H7 yang patogenik gagal dikesan.

Kata kunci: Escherichia coli, Escherichia coli O157: H7, sayur-sayuran organik, ujian kecenderungan antibiotik, MPN-PCR

CHAPTER 1

INTRODUCTION

Organic produces are vegetables and fruits grown and harvested without using any synthetic fertilizers and pesticides. Besides that, synthetic methods in producing organic products such as applying irradiation and artificial ripening of fruits are prevented in organic agriculture (FAO, 2013). Instead, organic farming adapts certain systems whereby soil is manipulated to be remained fertile for longer period and pests control can be done via natural means (FAO, 2013). Recently, the demand and interest for organic produces have been increasing as they are believed to be safer and healthier to consumers. Furthermore, researchers had also recommended that people should consume at least five servings of vegetables and fruits per day to maintain their health (James, 2006). However, organic produces may still contain natural contaminants from the environment such as soil, water and livestocks' manure (James, 2006). Since most of the organic fertilizers are made up of animal manure, this provides risk that pathogenic bacteria can be introduced to organic plants through spreading of manure in the environment (Mukherjee *et. al.*, 2004). According to a study performed by Beuchat and Ryu (1997), bacteria can be introduced to the fresh produces in the farm or during any stages of handling the produces (James, 2006). For example, enterobacteriaceae such as *Escherichia coli* (*E. coli*) which can be found naturally in the soil, water, animals and plants. Hence, Sivapalasingam *et al.* (2004) stated that they were not surprised for the occurrence of produce-related foodborne diseases that has been increasing during the past three decades (Annous *et al.*, 2009).

E. coli is common enteric bacteria that are found in the gastrointestinal tracts of humans and animals (CDC, 2012a). Most of them are harmless towards the hosts whereas some of them can cause diseases. Pathogenic *E. coli* normally causes diarrhea and thus they

are also known as diarrheagenic *E. coli.* According to Buchanan and Doyle (1997), there are six classes of diarrheagenic *E. coli* recognized. These includes Shiga-toxin-producing *E. coli* (STEC) or enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and diffusely adherent (DAEC). In this study, only EHEC known as *E. coli* O157: H7 was investigated.

E. coli O157: H7 is the most commonly isolated pathogenic strain of *E. coli* (CDC, 2012a). It causes wide outbreak of infections in the world every year. They are responsible for many types of foodborne diseases such as bloody diarrhea and hemolytic uremic syndrome (HUS) (CDC, 2012a). However, even though there were a lot of infection outbreaks caused by *E. coli* over the years, it has not been confirmed that fresh produces contain *E. coli* O157: H7 as most of the experiments conducted failed in isolating the target strain. According to investigation done by Mukherjee *et al.* (2004), they also, could not isolate any *E. coli* O157: H7 from 40 farms in Minnesota.

So far, there is no reported outbreak of *E. coli* in Malaysia. However, the government and health department of the country had issued warnings to citizens that had visited Europe countries in year 2011 to be investigated at nearby health centers. This is due to an outbreak of *E. coli* believed to be originated from cucumbers from Germany that year. The outbreak had caused 15 deaths in Germany and hundreds of illness (The Star, 2011). It was reported that the cucumbers were contaminated by EHEC bacteria, thus able to cause diseases such as HUS and bloody diarrhea.

There are over 100, 000 cases per year reported to be caused by EHEC (DuPont, 2007). Among these, *E. coli* O157:H7 is responsible for 73, 000 cases with more than 2,000 hospitalizations and 60 deaths per year in United States (CDC, 2005). Normally outbreaks of

E. coli O157: H7 are caused by contamination of the food products such as fresh produces and undercooked ground beef by uncleaned irrigation water, animals' manures, uncleaned harvesting tools and the hygienic level of workers processing the food. Several studies have reported the presence of *E. coli* O157: H7 in some organic foods in Malaysia (Chang *et. al.*, 2013). Therefore, the purpose of this research was to detect the presence of *E. coli* and *E. coli* O157: H7 in raw organic vegetables in Kuching and Siburan areas. Another aim of this research was to determine the antibiotic susceptibility profile of the *E. coli* and *E. coli* O157: H7 isolates.

CHAPTER 2

LITERATURE REVIEW

2.1 Genus, Morphology and Features of *Escherichia coli*

Escherichia coli (*E. coli*) belongs to the family Enterobacteriaceae and genus *Escherichia.* It is a Gram-negative, non endospore-forming, motile and facultative anaerobic bacterium. Under the microscope, it is seen as straight rods with the size of 2.0 x 0.5 μm. When being plated on Eosin Methylene Blue (EMB) agar, the bacterium will form colonies of dark blue-black color with metallic green sheen (American Society for Microbiology, 2012).

E. coli can be found naturally in the gastrointestinal tract of human being and warm-blooded animals such as cattle and domestic livestock (ECDC, 2013). In general, most of the strains of this bacterium are beneficial towards the host. It helps to maintain the environment of the intestines by suppressing the growth of foreign bacteria (FDA, 2012). However, there are also some serotypes of *E. coli* which are harmful towards us such as *E. coli* O157: H7, *E. coli* O145 and *E. coli* O26 (CDC, 2012a).

2.2 History of *E. coli and E. coli O157: H7*

In year 1885, Theodor Escherich, a German pediatrician, discovered *E. coli* in normal individual's faeces and at that time, he named the bacterium as Bacterium *coli commune*. The name was given because the bacterium was found in the colon part of the people. However, in the later years, genus Bacterium had been eliminated and subsequently, Bacterium *coli commune* was renamed *Escherichia coli*, named after its pioneer discoverer.

One of the serotype called *E. coli* O157: H7 was first identified and recognized as pathogenic *E. coli* in year 1982 when an outbreak was caused by it (Riley *et al.,* 1983). This serotype was found to cause hemorrhagic colitis and leads to Hemolytic uremic syndrome (HUS). After 11 years, another outbreak was caused by *E. coli* O157: H7 and it infected multiple states in the United States. Researchers by then discovered the connection between the bacterium with undercooked ground beef patties from a fast-food restaurant (Bell *et. al.,* 1994). A year later, the bacterium became widely known and people were warned against it.

2.3 Enterohermorrhagic *E. coli* (EHEC) or Shigatoxigenic *E. coli* (STEC)

E. coli O157: H7 is the predominant serotype under EHEC or STEC. It was named according to the O and H antigen displayed on the outer membrane. The outer membrane of the bacterium consists of lipopolysaccharides (LPS) molecule including the O antigen. The antigen is encoded by *rfb* gene cluster (O'Brien *et al.*, 2005). Meanwhile, H antigen can be found on the flagella of the bacterium itself. It is encoded by *fli*C_h7 genes (O'Brien *et al.*, 2005). The presence of virulence genes such as stx_1 and stx_2 indicates that this bacterium is capable to produce Shiga toxin (Franz *et. al.*, 2007). This toxic can cause severe damages to the lining of gastrointestinal tracts (FDA, 2012). This serotype of the bacterium is thought to be the most common cause of bacterial diarrhea following *Salmonella* spp. Over the past years, it has been associated with unpasteurized dairy products, contaminated water and juices, fresh produces and also raw or undercooked ground beef meat (Boyce, 2012).

E. coli O157: H7 is pathogenic and can cause an acute disease known as hemorrhagic colitis (FDA, 2012). The symptoms of the disease include the occurrence of bloody diarrhea after few days of watery diarrhea, abdominal cramp and in some cases; fever and vomiting may also happen (Cohen and Giannella, 1992). Usually, the sickness will last for an average

of eight days (FDA, 2012). For some patients of hemorrhagic colitis, if they are not treated accordingly, there is a chance they may develop HUS. Some of the consequences that might occur with HUS are anemia, thrombocytopenia and even kidney failure (Boyce, 2012). Furthermore, it may lead to severe illness such as nerve or brain damage, or even death in older patients (Boyce, 2012). Other symptoms of disease caused by infection of *E. coli* O157: H7 are pneumonia and respiratory diseases (CDC, 2012a). *E. coli* O157: H7 is more significant if compared to other bacteria that cause gastrointestinal diseases because it can cause infection even in low doses and affecting people with any age group, with young kids and old people at greater risk. Besides, it is also found out to be more acid tolerant than other bacteria (Buchanan and Doyle, 1997).

2.4 Foodborne Outbreaks related to *E. coli* O157: H7

EHEC or STEC causes approximately 100, 000 cases per year according to DuPont (2007). According to the Centers for Disease Control and Prevention (CDC) (2005), *E. coli* O157: H7 causes 73,000 diseases with at least 2,000 hospitalizations and 60 deaths per year in the United States. Produce-related outbreaks were first reported in 1991 and it has been emerging as an infection vehicle until nowadays (Rangel *et al.,* 2005). According to the epidemiology study conducted by Rangel *et al.* (2005), it was reported that most produce-associated outbreaks are linked to vegetables such as lettuce, salad, coleslaw and sprouts. In Japan, an *E. coli* O157 outbreak linked with raw radish sprouts was reported and it had infected 12, 000 people with 12 deaths (Michino *et al.*, 1999).

Recently in September 2006, CDC reported a multistate outbreak associated with fresh spinach caused by this bacterium. The outbreak spread throughout 26 states of the

United States, Canada and even New Mexico. The consumption of freshly packaged baby spinach was the cause and 205 cases of illness and 3 deaths were reported (CDC, 2006).

Besides that, in December 2012, another report was lodged by CDC on another outbreak affecting 5 states of US caused by the same bacterium serotype and associated with organic spinach and spring mix blend. It infected 33 persons in which 13 of them were hospitalized and 2 of them diagnosed with HUS. Fortunately, no death was reported during the outbreak (CDC, 2012b).

2.4.1 Transmission of Bacteria into Food

For STEC, there are three routes of transmission which are through food, person-to-person and from the environment such as contaminated water and animals (DuPont, 2007). Fecal-oral route mostly occur at nurseries, and some in schools, housing communities and public facilities (Rangel *et al.,* 2005). Fresh produces such as vegetables and fruits are susceptible to contamination of the bacterium. Likewise, organic farms are also at risks of contamination with the bacterium as various studies have reported organic produces are more likely to be contaminated with the bacterium than conventional farms. However, it has yet to be proven whether the statement is true (Mukherjee *et al.*, 2004). So far, there is only one confirmed report regarding the consumption of organic vegetable which led to foodborne outbreak (Tschape *et al.*, 1995). All produces have equal chances to be contaminated in fields by animal's manure, irrigation water or during processing, harvesting, transporting and storing (Rangel *et al.*, 2005). Cattle, bovine and domestic livestock manure had been used as fertilizer in growing produces. However, it is known that animal manure may contain gastroenteritis such as *E. coli, Salmonella, Mycobacterium paratuberculosis* and *Listeria* spp. (Pell, 1997). An experiment done by Soloman *et al.* (2002) has also demonstrated that *E. coli*

O157: H7 can enter through the root system of lettuce plant and finally be found in the edible parts of the lettuce (Solomon *et al.*, 2002)

To minimize the occurrence of such bacteria in the manure, a method called composting is introduced to the farmers. It is believed that composting the manure before using the manure directly as fertilizer can help to remove large number of bacteria in it as heat is generated over time (Hussong *et al.*, 1985).

2.5 Organic Farming

Organic produces are produce that are grown without the use of any synthetic fertilizer, herbicides and pesticides. In an organic farm, it is common to find animal manures composted to be used as fertilizers. Any synthetic methods such as irradiation and introducing artificial ripening of fruits are not allowed in an organic farm. Apart from that, organic farming also adapts certain systems whereby soil is being manipulated to be fertile for longer period and pests control can be done in natural ways (FAO, 2013).

There are quite a number of reports on the occurrence of foodborne pathogens in organic produces. Tshape *et al.* (1995) reported that green butter made with organic parsley contaminated with verotoxinogenic *Citrobacter freundii* was the cause of a severe gastroenteritis followed by HUS outbreak in a nursery school and kindergarten in Germany. In year 2001, McMahon and Wilson had conducted a study on the occurrence of foodborne pathogens in 86 commercially available organic vegetables in Northern Ireland. Thirty-four percent of the tested samples were found to be contaminated with *Aeromonas* spp. (McMahon and Wilson, 2001).

In Malaysia, Chang *et al.* (2013) had reported the low contamination of *E. coli* O157: H7 in organic four- winged beans and white radish sold at supermarkets and retail groceries respectively. The highest prevalence of *E. coli* O157: H7 in their study was found in organic chickens where 40% of the tested samples (n= 20) harboured the bacteria.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sample collection

A total of 51 samples made up of seven types of organic vegetables (Table 1) were collected randomly from four organic farms in Kuching and Siburan, Malaysia. Among all four organic farms, only one is certified by the Malaysian Organic Scheme (SOM) and the others claimed to be practicing organic farming. Samples were picked randomly in three different sites at the farm and kept in sterile ziplock bags labeled. The samples were transported in an ice box to the laboratory. Analysis of the samples was conducted within 24 hours upon collection of samples.

Table 1. Organic vegetables types and number of samples examined

English name	Scientific name	Total number of samples
Romaine lettuce	*Lactuca sativa*	9
Chinese broccoli	*Brassica oleracea*	9
Tomato	*Lycopersicon esculentum*	6
Salad (Sorrel)	*Rumex acetosa*	6
Chinese cabbage	*Brassica rapa*	12
Other leafy greens (Spinach and Mustard)		9
TOTAL		**51**

3.2 Sample Processing

3.2.1 Sample Enrichment

Twenty five grams of the organic vegetable samples were enriched in 225 ml of Luria-Bertani (LB) Broth and homogenized in a sterile stomacher bag for 2 minutes. Then, the mixture was incubated at 37°C for 24 hours.

3.2.2 Serial Dilutions

The incubated sample mixture was subjected to ten-fold serial dilutions. One ml of the sample mixture was diluted with 9 ml of sterile saline solution (0.85% NaCl). Then, 1 ml of the aliquot from first dilution was transferred to second test tube with dilution factor of 10^{-2}. The same procedures were carried out until dilution factor of 10^{-7}.

3.2.3 Most Probable Number (MPN)

One ml of aliquot from dilution 10^{-1} until 10^{-3} were transferred into triplicate MPN tubes containing 9 ml of LB. The tubes were then incubated at 37°C for 24 hours. A tube containing only LB was prepared and used as a control. On the next day, test tubes that showed turbidity were then subjected to DNA extraction and PCR for the detection of *rfb*O157, *fliC*$_h$*7*, Shiga toxin I and II genes.

3.2.4 Colony-Forming Unit (CFU)

Meanwhile, 100 μL of the aliquot from dilution 10^{-3} until 10^{-7} were plated on Eosin-Methylene Blue (EMB) agar. The plates were incubated at 37°C for 24 hours. After incubation, green metallic sheen colonies forming on the EMB agar were counted. The isolates were then stored on LBA.

3.3 DNA Extraction

DNA extraction was conducted using the boil cell method as described by Apun *et al.* (2011) with minor modifications. One ml of aliquot from each overnight MPN tube was centrifuged at 13,000 rpm for 5 minutes. Then, the supernatant was discarded and the pellet was resuspended with 200 µL of sterile distilled water. The mixture was vortexed slightly to make sure that remaining pellet was dissolved. Following that, the mixture was boiled for 20 minutes and immediately cooled for 20 minutes at -20°C. After cooling, the mixture was centrifuged again at 13,000 rpm for 3 minutes. Final supernatant containing DNA was transferred to a new microcentrifuge tube and store at -20°C until needed for PCR assay.

3.4 PCR Amplification

Four pairs of primers (*SltI, SltII, rfbE, fliC$_h$7*) targeting Shiga toxin I and II genes, O157 antigen gene and H7 antigen gene were used in the PCR amplification for detection of pathogenic *E. coli* O157: H7. PCR amplification was performed in 25 µL reaction mixture consisting 2.5 µL of 10X PCR buffer, 1.25 µL of 10 mM deoxynucleoside triphosphate (dNTP) mix, 2.5 µL of 25 mM $MgCl_2$, 0.50 µL of 5 U/µL *Taq* Polymerase, 10 µL of template DNA and 0.5 µL of each primer pairs (*SltI-F/SltI-R, SltII-F,SltII-R, rfb-F/rfb-R, fliC$_h$7$_7$-F/ fliC$_h$7-R). E. coli* O157: H7 reference strains EDL 933 was also included in the PCR assay as positive control. The thermal cycling conditions were as stated in Table 2.

Table 2. Cycling Conditions for PCR Amplification

Process	Temperature (°C)	Duration (s)	Cycle(s)
Initial Denaturation	94	300	1
Denaturation	94	30	35
Annealing	59	60	
Extension	72	60	
Final Extension	72	420	1

3.5 Agarose Gel Electrophoresis (AGE)

Amplified PCR products were subjected to AGE. Two percent agarose gel was prepared using 1X Tris-Borate EDTA (TBE) buffer. Four microlitre of PCR product was mixed with 1 μL of 6X loading dye and then loaded into the wells prepared. After loading the PCR products in the well, 3 μL of 100 bp marker was loaded into the well as an indicator of the band size. After that, the PCR products were electrophoresed at 85 V for 1 hour. Then, the gel was stained in Ethidium Bromide (EtBr) for 20 minutes before it was viewed under an ultraviolet transilluminator and photographed using gel documentation system (AlphaDigiDoc RT). The expected sizes of amplicons for *rfb*O157, *fliC*$_h$7 and Shiga toxin-producing genes were 292 bp, 625 bp, 210 bp and 484 bp respectively.

3.6 Antimicrobial Susceptibility Test (AST)

AST was performed using disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI). First, the bacterial isolate from LBA was revived by incubating overnight in LB at 37°C. The viability was tested by obtaining OD_{600} value ranging from 0.8 to 1.0. After that, sterile cotton bud was used to transfer and spread the bacteria on Mueller-Hinton Agar (MHA) plate. Antibiotics tested include ampicillin